Hydrops, fetal pleural effusions and chylothorax in three patients with CBL mutations

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Abstract: Fetal hydrops, fetal pleural effusions, hydrothorax, and chylothorax, may be associated with various genetic disorders, in particular with the Noonan, cardio-facio-cutaneous and Costello syndromes. These syndromes, collectively called RASopathies, are caused by mutations in the RAS/MAPK pathway, which is known to play a major role in lymphangiogenesis. Recently, germline mutations in the Casitas B-cell lymphoma (CBL) gene were reported in 25 patients and of these, 20 had juvenile myelomonocytic leukemia (JMML). The disorder was named "CBL syndrome" or "Noonan syndrome-like disorder with or without juvenile myelomonocytic leukemia" (NSLL). To date, prenatal abnormalities have not been reported and it is still debated whether the CBL syndrome falls into the category of a RASopathy, or represents a different entity. Here we report on three unrelated patients with CBL mutations manifesting with hydrops fetalis, fetal pleural effusions and/or congenital hydro-/chylothorax. Our findings further connect the CBL syndrome with the RASopathies.

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CLINICAL REPORT

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Hydrops, Fetal Pleural Effusions and Chylothorax in Three Patients With *CBL* Mutations

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Key words: congenital hydrothorax or chylothorax; hydrops; Noonan syndrome-like disorder with or without juvenile myelomonocytic leukemia (OMIM #613563); *CBL* gene; RASopathy

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INTRODUCTION

Noonan syndrome (NS) is a genetically heterogeneous disorder characterized by short stature, congenital heart defects, characteristic facial features, and variable developmental delay. Several genes encoding components and modulators of the RAS/MAPK pathway have been shown to cause NS and related disorders collectively called RASopathies [Zenker, 2011]. In 2010, heterozygous germline mutations of the Casitas B-cell lymphoma (*CBL*) gene were independently discovered in individuals with features of NS who had no mutations in previously known disease genes [Martinelli et al., 2010], and among patients with juvenile myelomonocytic leukemia (JMML), some of which displayed congenital anomalies of the RASopathy spectrum [Perez et al., 2010; Niemeyer et al., 2010]. Clinical features of the *CBL* mutation-associated disorder included short stature, facial dysmorphism, hyperpigmented nevi, pterygium colli, cardiovascular abnormalities, pectus excavatum, joint laxity, cubitus valgus, neonatal feeding problems, and attention deficit hyperactivity disorder [Perez et al., 2010; Martinelli et al., 2010]. Some patients also had abnormalities suggesting an underlying vasculopathy, such as childhood-onset arterial hypertension, acquired cardiomyopathy, and Takayasu arteriitis in one case [Niemeyer et al., 2010].

As a common molecular pathophysiology, mutations causative for the RASopathies have been shown to hyperactivate or dysregulate the RAS/MAPK pathway signaling. The same appears to be true for germline *CBL* mutations which are suggested to act through impaired receptor ubiquitylation leading to dysregulated signaling through RAS [Martinelli et al., 2010]. The new syndrome was designated

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Noonan syndrome-like disorder with or without juvenile myelomonocytic leukemia (NSLL) or CBL syndrome (OMIM 613563).

Congenital chylothorax is an uncommon disorder (incidence 1/10,000–15,000 births) and the most common cause of pleural effusions in newborns [Fernández Alvarez et al., 1999]. The accumulation of lymphatic fluid that becomes milky after the onset of enteral nutrition is thought to result from abnormal lymphatic vessel development. Effusions often resolve with adequate treatment in the first weeks or months. In the majority of cases the etiology remains unknown. Congenital chylothorax was previously described with chromosomal conditions including the Down [Hamada et al., 1992 Turan et al., 2001; Manghat et al., 2004; Kabbani et al., 2005] and Turner syndromes [Moerman et al., 1993], and with Mendelian disorders including the Diamond–Blackfan [Lazarus and McCurdy, 1984], Adams–Oliver [Farrell et al., 1993], Noonan [Prasad et al., 2002; Chen et al., 2009], and Opitz G/BBB syndromes [Funke et al., 2006]. Fetal chylothorax may progress to hydrops fetalis, which may reflect a more generalized lymphatic dysplasia.

Here we report on three patients with *CBL* mutations, who had a pre- and post-natal hydro-/chylothorax and/or hydrops, thus expanding the clinical phenotype of the NSLL/CBL syndrome and further highlighting the phenotypic overlap with the other RASopathies.

CLINICAL REPORTS

Patient 1

Patient 1 (Fig. 1A–C) was born to a healthy 35-year old G1 P1 and a nonconsanguineous 36-year old father. Family history was unremarkable. Data on nuchal translucency were not available. Fetal pleural effusions were noted at 21 weeks of gestation. There was no evidence of an infectious cause. Intrauterine transfusion was performed due to a suspected fetal anemia, mildly increasing the hemoglobin from 13.9 to 15.1 g/dl (reference range: 12–18 g/dl). The hydrothorax did not improve and at week 27, pleural effusions were relieved by bilateral thoracocentesis. At 31 + 5 weeks of gestation, placental insufficiency necessitated premature delivery. Birth weight was 1,410 g (10–25th centile), length 39 cm (10–25th centile), and occipitofrontal circumference (OFC) 29.5 cm (10th centile). Grade 4 respiratory distress syndrome, grade 1 intraventricular hemorrhage and supravalvular pulmonary stenosis were diagnosed. By thoracocentesis, a milky fluid was obtained and a chylothorax was diagnosed. She remained critically ill during her first 6 months of life. The chylothorax was drained three times and she was fed with medium chain triglyceride (MCT) diet. At age 6 months, her length was 57 cm (−3.9 SD) and weight 3,410 g (−5.3 SD). The pleura was adhered using OK-432 (Picibanil®) and thereafter the effusions did not recur. At age 9 months the chylothorax had completely resolved. Subsequently her condition gradually improved. Development was severely delayed. She started to walk at age 35 months. Neuropediatric evaluation at 3 years indicated a developmental age of 12½ months, but then her general condition and development improved. She was first seen by a clinical geneticist (O.B.) at age 3½ years.

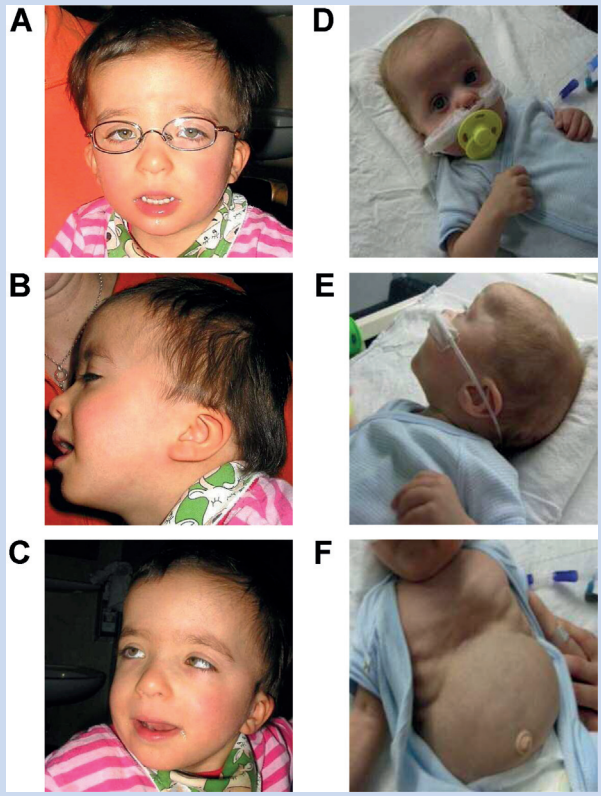

FIG. 1. (A–C) Female Patient 1 at age 3½ years; note (A) high forehead, hypertelorism, and ptosis, (B) low-set ears, and (C) downslanting palpebral fissures. (D–F) Male Patient 3 at age 5 months, note (D) prominent forehead and downslanting palpebral fissures, (E) low-set ears, (F) pectus excavatum and prominent abdomen.

Body height was 98 cm (25–50th centile), weight was 12 kg (3–10th centile), and OFC was 47 cm (3rd centile). She had thin and sparse hair, high forehead, hypertelorism, mild ptosis, downslanting palpebral fissures, and low-set ears (Fig. 1A–C). She also had esotropia, astigmatism, and hyperopia corrected by glasses. She used two-word-sentences. Her gait was broad-based and unsteady but she could walk safely with bilateral ankle ortheses. Ultrasound showed mild supravalvular pulmonary stenosis, mild splenomegaly, cholecystolithiasis, and bilateral hip dysplasia. At 5¼ years she began to use three-word-sentences, but could not hop and jump or perform a "one-leg-stand". Height was 109 cm (25th centile), weight was 17 kg (10–25th centile), and OFC was 48.5 cm (3rd centile). When last seen at age 6 years, she had severe hypotonia and strabismus. Her spleen was enlarged and palpable 3–4 cm below the coastal arch. She consistently wore compression stockings for lymphedema of the legs. There had been no swelling of her genitalia. Her hip dysplasia was re-evaluated; the left leg was found to be mildly shorter and osteotomy was postponed due to the chronic lymphedema. She had made major progresses in development, could walk stairs, could ride a bike with training wheels, and had begun speaking in whole sentences, naming colors, and counting.

Physio-, ergo-, and speech therapies were continued and primary school enrollment was postponed for a year.

Amniocentesis and karyotyping at age three months had been normal. At age 3½ years, her congenital chylothorax, pulmonary stenosis and facial appearance were considered suggestive of a RASopathy. Using blood DNA, molecular, molecular genetic analysis of *PTPN11*, *SOS1*, *RAF1*, *KRAS*, *NRAS*, *SHOC2*, and *CBL* was performed showing a de novo mutation c.1100A>C (predicting p.Q367P) in *CBL* exon 8 (Fig. 2A). The germline nature of the mutation was confirmed by testing a saliva sample.

Patient 2

Patient 2 was born without complications at 36 weeks of gestation to unrelated European parents, which apart from maternal C1 esterase deficiency were healthy. Fetal ultrasound had showed a left-sided hydrothorax, mild skin edema, and ascites (hydrops fetalis), as well as hepatosplenomegaly and polyhydramnios without evidence for infectious, toxic or immune-mediated disease. Data on nuchal translucency were not available. Birth weight was 3,165 g (75–90th centile), length 50 cm (50–75th centile), and OFC 34.5 cm (50–75th centile). Apgar scores were 3^1, 6^5 and umbilical pH was 7.20. Due to cyanosis he received bag ventilation and was intubated after 15 min. At the same time the left sided hydrothorax was punctured with placement of drainage. At age 3 weeks, the drainage was removed, and the hydrothorax did not recur. Echocardiography at age 4 weeks revealed mild valvular pulmonary stenosis. Testes were descended but showed mild hydroceles. Torticollis and feeding problems were noted neonatally and treated with physiotherapy and gastric tube feeding followed by gastrostomy tube insertion until age 4 years. Despite the clinical diagnosis of Noonan syndrome, extensive metabolic work-up and bone marrow cytology were performed at age 1 year due to persisting splenomegaly, frequent infections, generalized muscular hypotonia, failure to thrive and significant psychomotor retardation, and showed no abnormalities. Since age 1, length and weight were below the 3rd centile, with relative macrocephaly. Sitting age was 21 months, walking age was three years and he spoke single words at age 2 years. Cranial MRI with proton spectroscopy at age 2 affirmed some expansion of subarachnoid spaces, delayed myelinisation and hypoplasia of olfactory bulbs and optic chiasm, and normal results of spectroscopy. At age 4, a myopathy was assumed due to severe muscular hypotonia, diminished strength, reduced muscle mass, and Gower's sign. At age 7, persisting splenomegaly, long-lasting

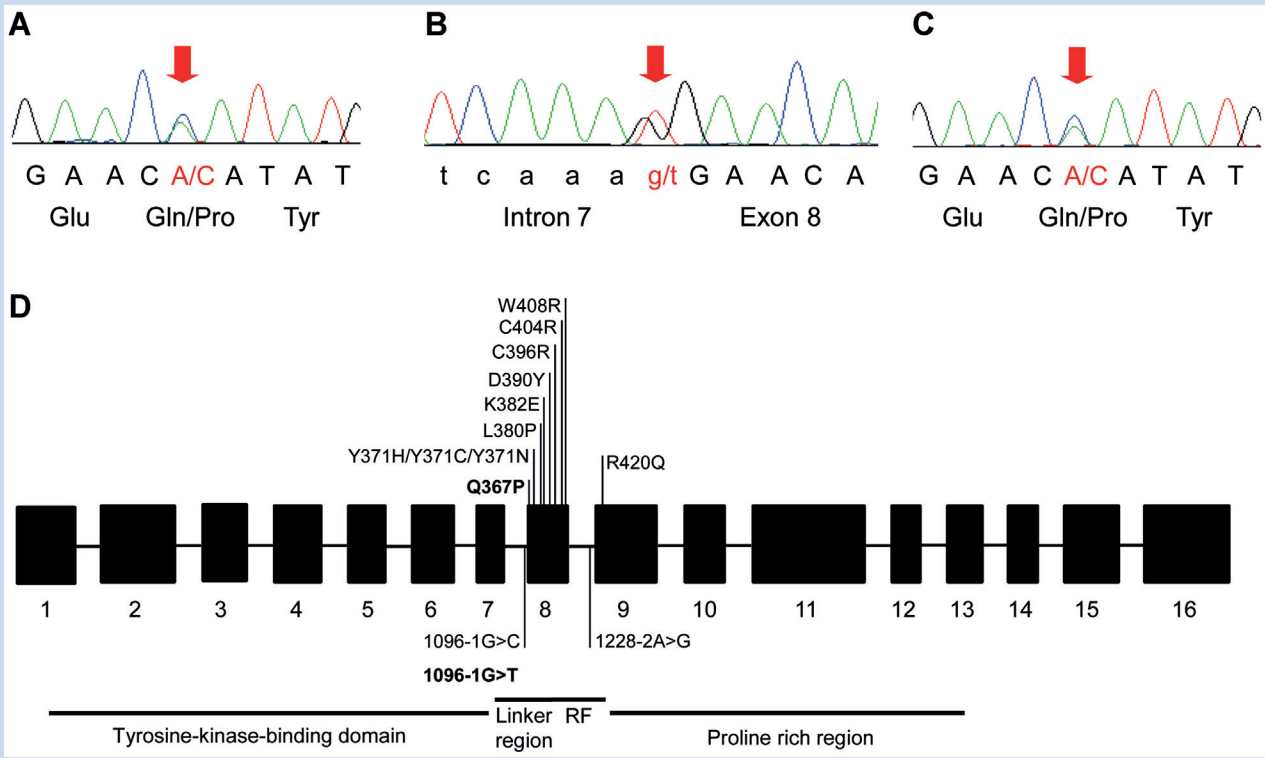

FIG. 2. (A–C) Electropherograms of Patients 1, 2, and 3, respectively. Note identical heterozygous *CBL* mutations c.1100A>C in Patients 1 and 3 and splice site mutation c.1096–1G>T in Patient 2. (D) Locations of 15 *CBL* mutations in 28 unrelated patients with NSLL with or without JMML. Mutations identified in this study, Q367P (Patients 1 and 3) and 1096–1G>T (Patient 2) are shown in bold. Exons are numbered and drawn to scale; introns and UTRs are not drawn to scale. Note that all mutations alter exons 8–9, corresponding to the linker and RING finger (RF) domains.

and easy bruising lead to the diagnoses of immunoglobulin-responsive thrombocytopenia, diminished factors II, IX, X, and XII, and microcytic hypochromic anemia. C1 esterase deficiency was excluded.

At age 7, 10 months weight was 18.3 kg (2nd centile), height 118.4 cm (4th centile), and OFC 50.7 cm (6th centile). He had bilateral ptosis, downslanting palpebral fissures, hypertelorism, epicanthal folds, lateral flaring eyebrows, low-set backwards rotated ears, low posterior hair line, asymmetric ear lengths, and a general hypotonic expression. He also had broad thorax, mild pectus excavatum and carinatum, bilateral pes planovalgus, low muscle mass, broad low-set thumbs, bilateral single palmar crease, fetal pads, and broad first toes. His knees and lower legs were hyperpigmented. At age 11, he was treated with corticosteroids and tranexamic acid for persistent splenomegaly, frequent epistaxis, and spontaneous bleedings. Lymphedema of the lower limbs had never been observed. He attended a regular school with special education, was able to read and write, but could not ride a bike or jump on one foot. He had a pleasant nature and good social interactions.

As his symptoms and facial appearance were suggestive of a RASopathy, molecular genetic analysis of *PTPN11*, *SOS1*, *RAF1*, *NRAS*, *BRAF*, *MEK1*, *MEK2*, and *CBL* was performed showing a de novo *CBL* mutation c.1096–1G>T at the splice acceptor site of exon 8 (Fig. 2B). No DNA from saliva or buccal mucosa was available to confirm the germline origin of the mutation.

Patient 3

Patient 3 (Fig. 1D–F) is the second child of nonconsanguineous parents. Nuchal translucency measurement at 12 weeks was normal (1.3 mm), but the pregnancy was subsequently complicated by polyhydramnios. Moderate fetal hydrops and bilateral pleural effusions, worse on the right, and accompanied by mediastinal shift, were identified at 31 weeks gestation, necessitating premature delivery. Hemoglobin at birth was 18 g/dl (reference range: 13.5–23 g/dl), suggesting that the fetal hydrops had not been caused by fetal anemia. Birth parameters were weight 2,190 g (90th centile), length 42 cm (50th centile), and head circumference 30.5 cm (50–90th centile). Postnatally, an atrial septal defect (ASD), right inguinal hernia, bilateral undescended testes, and hepatosplenomegaly were detected. A blood smear at age 3 weeks showed monocytosis, and bone marrow aspirate was indicative of JMML. Renal ultrasound and coagulation studies were normal. At 7 months of age feeding was exclusively via naso-jejunal tube (NJT) due to severe feeding difficulties and gastro-esophageal reflux. He had an ongoing oxygen requirement despite the resolution of pleural effusions and hydrops. Bilateral lung opacities were identified on chest radiograph, and lung biopsy showed features of lymphangiectasia. When assessed at 17 months old, he was able to sit with support, and rolled inconsistently. He banged objects together, waved, and babbled but did not have any recognizable words. When reviewed at 20 months of age, his weight was 9.6 kg (10–30th centile), length was 77 cm (<3rd centile) and head circumference was 46 cm (10th centile). He had prominent forehead, downslanting palpebral fissures, low-set ears, and pectus excavatum (Fig. 1D–F). There was hypotonia and motor milestones were delayed. He is on continuous feeds via a percutaneous gastrostomy (PEG) and still has an ongoing oxygen requirement. There had been no lymphedema of the lower limbs.

SNP microarray and *PTPN11* sequencing returned normal results. *CBL* sequencing showed a de novo missense mutation c.1100A>C (predicting p.Q367P) (Fig. 2C). The germline nature of the mutation was confirmed by testing a buccal swab sample.

DISCUSSION

Herein we report on three patients, who were screened for mutations in several RAS/MAPK pathway genes because of the clinical suspicion of a RASopathy and were found to carry de novo mutations in the *CBL* gene. *CBL* mutations have been described as somatic mutations in various leukemias [Loh et al., 2009; Schnittger et al., 2012] and as germline mutations in patients showing variable developmental abnormalities with or without JMML. Most of the *CBL* mutations found as germline and somatic events are located in the linker and ring finger domain (RF) encoded by exons 7–9 [Schnittger et al., 2012] (Fig. 2D). Patients 1 and 3 had an identical mutation in exon 8, c.1100A>C (p.Q367P), which had been previously identified in a patient with developmental delay, cafe-au-lait macules, enlarged left atrium, and transient chaotic ventricular dysrhythmias [Martinelli et al., 2010] and in a patient with primary lymphedema and teratoma [Hanson et al., 2014]. The mutation detected in Patient 2 (c.1096–1G>T) was similarly described (c.1096–1G>C) in a patient with developmental delay, cryptorchidism, juvenile xanthogranuloma (JXG), and JMML [Niemeyer et al., 2010].

The present patients all had craniofacial features compatible with a Noonan-like syndrome, including congenital heart defects typical of RASopathies, short stature, and delayed development. We assume that perinatal complications and/or JMML may have contributed to their developmental delay. Only 14 (56%) of the 25 previously reported NSLL patients were diagnosed with developmental delay (Table I); 11 patients showed normal development and in one girl with developmental delay in childhood had caught up at age 15 years [Martinelli et al., 2010].

It has been increasingly recognized that the RAS-MAPK pathway plays a major role in the signaling of lymphangiogenesis [Coso et al., 2014]. Remarkably, our patients all had a history of prenatal pleural effusions, congenital hydro-/chylothorax, and/or hydrops fetalis. While such complications have not previously been described in individuals with CBL germline mutations, they belong to a spectrum of prenatal abnormalities that are typically associated with the RASopathies. The most frequent prenatal findings in Noonan syndrome and related disorders include increased nuchal translucency and cystic hygroma, but pleural effusions, hydrops, and lymphedema have also been reported [Witt et al., 1987; Witters et al., 2002; Yoshida et al., 2004; Baldassarre et al., 2011]. These abnormalities may be regarded as a continuous spectrum caused by impaired fetal lymphatic drainage probably related to lymphatic dysplasia or delayed maturation of lymphatic vessels. The lymphedema of the feet and legs in Patient 1 and in another patient with the same c.1100A>C mutation [Hanson et al., 2014] points to an underlying abnormality of the lymphatic system [Witt et al., 1987; Roberts et al., 2013] and further documents the pathophysiological relationship of the CBL syndrome with the Rasopathies. Sponta-

TABLE I. Clinical Features of 28 Patients With Germline *CBL* Mutation and NSLL With or Without JMML

Clinical features	Martinelli et al., 2010	Perez et al., 2010	Niemeyer et al., 2010	Hanson et al., 2014	This study	Total cases
Short stature, postnatal onset	1/4	3/3	4/17	0/1	2/3	10/28
Microcephaly	0/4	2/3	—[a]	0/1	0/3	2/11
Dysmorphic features[b]	4/4	3/3	—[a]	1/1	3/3	11/11
Thorax abnormality[c]	3/4	1/3	—[a]	1/1	2/3	7/11
Cafe-au-lait spots	2/4	2/3	4/17	0/1	0/3	8/28
Cardiac malformations	3/4	0/3	—[a]	1/1	3/3	7/11
Cardiomyopathy	0/4	0/3	2/17	0/1	0/3	2/28
Hypertension	0/4	0/3	4/17	0/1	0/3	4/28
Developmental delay	3/4	1/3	9/17	1/1	3/3	17/28
Pleural effusion	0/4	0/3	—[a]	0/1	3/3	3/11
JMML	0/4	3/3	17/17	0/1	1/3	21/28
Lymphedema	—[a]	—[a]	—[a]	1/1	1/3	2/4
Teratoma	0/4	0/3	1/17	1/1	0/3	2/28
Cryptorchidism (males)	0/1	0/0	3/9	0/0	1/2	4/12

[a]Data not reported in the study.
[b]High forehead, hypertelorism, ptosis, down slanting palpebral fissures, low set ears, prominent philtrum, and short neck.
[c]Widely spaced nipples, pectus excavatum, and pectus carinatum.

neous resolution is common, but in some cases lymphedema and lymphatic/chylous effusions may persist until and beyond birth. As the outcome of fetal hydrops is generally poor, Witters et al. [2002] found it noteworthy that in a patient with Noonan syndrome, hydrops resolved spontaneously. However, the outcome of Noonan syndrome-related hydrops is not always favourable and lethality of Noonan syndrome-associated hydrops, particularly intrauterine lethality, may be largely underestimated [Yoshida et al., 2004; Lee at al., 2009]. Recently, a patient with hydrops fetalis, severe ascites, pleural effusion, and the p.S2G SHOC2 mutation was described [Gargano et al., 2014]. He died two days after he was born at 30 5/7 weeks gestation due to the severity of his symptoms. Previously described patients with a CBL germline mutation were selected either through their Noonan-like features or through being affected by JMML. Taking these cohorts together, it can be concluded that the clinical phenotype associated with CBL mutations generally includes developmental and physical abnormalities that overlap with Noonan syndrome or the RASopathy pattern of anomalies [Perez et al., 2010; Martinelli et al., 2010; Niemeyer et al., 2010; Digilio et al., 2011].

This is in line with the experimental findings showing that the mutated protein has a similar effect on the RAS/MAPK pathway as Noonan syndrome-associated mutations. However, it is likewise apparent that the Noonan-like features are more variable, and not all patients would be classified as having Noonan syndrome on a clinical basis alone. Therefore, it was debated whether this syndrome should be classified as Noonan-like or rather as a distinct "CBL syndrome". The clinical observations presented here further support the similarities between the *CBL* mutation-associated syndrome and Noonan syndrome, as we can document that these conditions also share a typical (albeit not specific) pattern of prenatal abnormalities. It is obvious that at least part of the typical craniofacial phenotype of Noonan syndrome (e.g., pterygium colli, low posterior hairline, and low-set ears) is the consequence of a fetal nuchal edema. Last not least, the presented cases underline that *CBL* mutations have to be included in the differential diagnosis of fetal pleural effusions, hydrops fetalis, and probably also fetal nuchal edema.

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